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## PATENT APPLICATION

### **BIOSYNTHESIS OF ISOPENTENYL PYROPHOSPHATE**

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## BIOSYNTHESIS OF ISOPENTENYL PYROPHOSPHATE

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### TECHNICAL FIELD

The present invention relates to the biosynthesis of isopentenyl pyrophosphate (IPP) and isoprenoids derived therefrom. More particularly, the invention relates to methods for biosynthesizing isopentenyl pyrophosphate, and to nucleic acid sequences, enzymes, expression vectors, and transformed host cells for carrying out the methods.

### BACKGROUND

Isoprenoids are compounds derived from the five-carbon molecule, isopentenyl pyrophosphate. Investigators have identified over 29,000 individual isoprenoid compounds, with new ones continuously being discovered. Isoprenoids are often isolated from natural products, such as plants and microorganisms, which use isopentenyl pyrophosphate as a basic building block to form relatively complex structures. Vital to living organisms, isoprenoids serve to maintain cellular fluidity and electron transport, as well as function as natural pesticides, to name just a few of their roles *in vivo*. Furthermore, the pharmaceutical and chemical communities use isoprenoids as pharmaceuticals, nutriceuticals, flavoring agents, and agricultural pest control agents. Given their importance in biological systems and usefulness in a broad range of applications, isoprenoids have been the focus of much attention by scientists.

Conventional means for obtaining isoprenoids include extraction from biological materials (e.g., plants, microbes, and animals) and partial or total organic synthesis in the laboratory. Such means, however, have generally proven to be unsatisfactory. For example, organic synthesis is usually complex since several steps are required to obtain the desired product. Furthermore, these steps often involve the use of toxic solvents, which require special handling and disposal. Extraction of isoprenoids from biological materials may also require toxic solvents. In addition, extraction and purification methods usually provide a low yield of

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the desired isoprenoid, as biological materials typically contain only small quantities of these compounds. Unfortunately, the difficulty involved in obtaining relatively large amounts of isoprenoids has limited their practical use. In fact, the lack of readily available methods by which to obtain certain isoprenoids has slowed down the progression of drug candidates through 5 clinical trials. Furthermore, once an isoprenoid drug candidate has passed the usual regulatory scrutiny, the actual synthesis of the isoprenoid drug may not lend itself to a commercial scale.

As a solution to such problems, researchers have looked to biosynthetic production of isoprenoids. Some success has been obtained in the identification and cloning of the genes involved in isoprenoid biosynthesis. For example, U.S. Patent No. 6,291,745 to Meyer et al. 10 describes the production of limonene and other metabolites in plants. Although many of the genes involved in isoprenoid biosynthesis may be expressed in functional form in *Escherichia coli* and other microorganisms, yields remain relatively low as a result of minimal amounts of precursors, namely isopentenyl pyrophosphate.

In an effort to address the lack of isopentenyl pyrophosphate, some investigators have attempted to increase isopentenyl pyrophosphate production. Croteau et al. describe in U.S. 15 Patent No. 6,190,895 the nucleic acid sequences that code for the expression of 1-deoxyxylulose-5-phosphate synthase, an enzyme used in one biological pathway for the synthesis of isopentenyl pyrophosphate. Low yields of isopentenyl pyrophosphate remain, however, since several more enzymes are needed to catalyze other steps in this isopentenyl 20 pyrophosphate biosynthetic pathway. Further, the reference does not address an alternative pathway for isopentenyl pyrophosphate biosynthesis, namely the mevalonate pathway.

Thus, the current invention is directed toward solving these and other disadvantages in the art by increasing the typically low yields associated with conventional synthesis of isopentenyl pyrophosphate and isoprenoids. Specifically, the current invention is directed 25 toward identification of new methods for the synthesis of isopentenyl pyrophosphate, as isopentenyl pyrophosphate represents the universal precursor to isoprenoid synthesis.

### SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to overcome the above-mentioned 30 disadvantages of the prior art by providing a method for synthesizing isopentenyl pyrophosphate

in a host microorganism, comprising the step of introducing into the host microorganism a plurality of heterologous nucleic acid sequences, each coding for a different enzyme in the mevalonate pathway for producing isopentenyl pyrophosphate.

It is another object of the invention to provide such a method wherein the plurality of heterologous nucleic acid sequences is contained in at least one extrachromosomal expression vector.

It is still another object of the invention to provide such a method wherein the isopentenyl pyrophosphate is further synthesized into an isoprenoid.

It is yet another object of the invention to provide such a method wherein the isoprenoid is selected from the group consisting of a monoterpene, sesquiterpene, diterpene, sesterterpene, triterpene, tetraterpene, and a steroid.

It is a further object of the invention to provide such a method wherein the plurality of heterologous nucleic acid sequences further comprises a DNA fragment coding for an enzyme capable of converting isopentenyl pyrophosphate to dimethylallyl pyrophosphate.

It is still a further object of the invention to provide a method wherein the host microorganism is a prokaryote.

It is an additional object of the invention to provide a method wherein the prokaryote is *Escherichia coli*.

Is it still another object of the invention to provide a method for synthesizing isopentenyl pyrophosphate in a host microorganism, wherein the method comprises introducing into the host microorganism an intermediate in the mevalonate pathway and at least one heterologous nucleic acid sequence, each said sequence coding for an enzyme in the mevalonate pathway necessary for converting the intermediate into isopentenyl pyrophosphate.

It is still a further object of the invention to provide DNA fragments, expression vectors, and host cells for carrying out the methods described herein.

Additional objects, advantages, and novel features of the invention will be set forth in part in the description that follows, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned through routine experimentation upon practice of the invention.

In one embodiment, the invention provides a method for synthesizing isopentenyl pyrophosphate in a host microorganism. The method comprises introducing into a host microorganism a plurality of heterologous nucleic acid sequences, each coding for a different enzyme in the mevalonate pathway for producing isopentenyl pyrophosphate. As will be appreciated by those skilled in the art, the mevalonate pathway involves six enzymes. The pathway starts from acetyl-CoA, proceeds through the intermediate mevalonic acid, and results in isopentenyl pyrophosphate. Of course, additional nucleotide sequences coding for other genes may be introduced as well. In particular, nucleotide sequences coding for enzymes necessary in the production of specific isoprenoids may be introduced into the host microorganism, along with those coding for enzymes in the mevalonate pathway. Preferably, at least one extrachromosomal expression vector will be used to introduce the desired nucleic acid sequence(s), although more than one (e.g., two) different expression vectors may be used. In addition, the desired nucleic acid sequence(s) may be incorporated into the host microorganism's chromosomal material.

In another embodiment, the invention provides a method for synthesizing isopentenyl pyrophosphate in a host microorganism by introducing into the host microorganism an intermediate of the mevalonate pathway and one or more heterologous nucleic acid sequences. The introduced sequence or sequences each code for an enzyme in the mevalonate pathway necessary for converting the intermediate into isopentenyl pyrophosphate. Thus, for example, if mevalonate is the introduced intermediate, the method requires introduction of nucleic acid sequences that code for the enzymes necessary to convert mevalonate into isopentenyl pyrophosphate, for example, the introduction of nucleic acid sequences coding for an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate, an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate, and an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate. Of course, other intermediates in the mevalonate pathway, along with the necessary nucleic acid sequences, may be introduced as well.

Although any host microorganism, e.g., a prokaryote or eukaryote, may be employed, it is preferred that a prokaryote such as *Escherichia coli* be used. Preferably, the host organism does not synthesize isopentenyl pyrophosphate through the mevalonate pathway, but rather

through the deoxyxylulose-5 phosphate (DXP) pathway. In this way, side reactions involving the intermediates of the mevalonate pathway are minimized, thereby enhancing the yield and efficiency of the present methods.

5 In another embodiment of the invention, DNA fragments, each coding for an enzyme in the mevalonate pathway, are provided in one or more expression vectors. Thus, for the mevalonate pathway, the DNA fragments include those that code for enzymes capable of: (a) condensing two molecules of acetyl-CoA to acetoacetyl-CoA, preferably the nucleotide sequence of SEQ ID NO 1; (b) condensing acetoacetyl-CoA with acetyl-CoA to form HMG-CoA, preferably the nucleotide sequence of SEQ ID NO 2; (c) converting HMG-CoA to mevalonate, preferably the nucleotide sequence of SEQ ID NO 3; (d) phosphorylating mevalonate to mevalonate 5-phosphate, preferably the nucleotide sequence of SEQ ID NO 4; (e) converting mevalonate 5-phosphate to mevalonate 5-pyrophosphate, preferably the nucleotide sequence of SEQ ID NO 5; and (f) converting mevalonate 5-pyrophosphate to isopentenyl pyrophosphate, preferably the nucleotide sequence of SEQ ID NO 6.

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15 In yet another embodiment, the invention provides expression vectors comprising the DNA fragments described above and elsewhere in the application, as well as host cells transformed with such expression vectors. The DNA fragments, expression vectors, and host cells transformed with the same expression vectors are useful in the present methods for synthesizing isopentenyl pyrophosphate.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B schematically illustrate the mevalonate pathway of isopentenyl pyrophosphate synthesis, along with enzymes involved and nucleic acid sequences coding for such enzymes.

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FIG. 2 is a graph illustrating the difference in the concentration of lycopene produced from natural levels of isopentenyl pyrophosphate in non-engineered *Escherichia coli* and from *Escherichia coli* engineered to overproduce isopentenyl pyrophosphate from a partial mevalonate-isoprenoid pathway, at different concentrations of mevalonate (Mev).

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FIG. 3 is a graph illustrating the difference in normalized lycopene concentration produced from natural levels of isopentenyl pyrophosphate in non-engineered *Escherichia coli*

and from *Escherichia coli* engineered to overproduce isopentenyl pyrophosphate from the complete mevalonate-isoprenoid pathway.

FIG. 4 is a graph illustrating the difference in amorphadiene concentration produced from natural levels of isopentenyl pyrophosphate in non-engineered *Escherichia coli* and from *Escherichia coli* engineered to overproduce isopentenyl pyrophosphate from a partial mevalonate-isoprenoid pathway.

FIG. 5 is a gas chromatographic spectrum illustrating the production of diterpene using ethyl acetate extracts from *Escherichia coli* engineered to produce isoprenoids from the artificial, modified MBIS operon (a partial mevalonate-isoprenoid pathway), and expressing a casbene cyclase.

For reference, FIG. 6 is the mass spectrum of the isoprenoid casbene.

#### DETAILED DESCRIPTION OF THE INVENTION

Before the invention is described in detail, it is to be understood that, unless otherwise indicated, this invention is not limited to particular sequences, expression vectors, enzymes, host microorganisms, or processes, as such may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to an "expression vector" includes a single expression vector as well as a plurality of expression vectors, either the same (e.g., the same operon) or different; reference to "microorganism" includes a single microorganism as well as a plurality of microorganisms; and the like.

In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

The terms "optional" or "optionally" as used herein mean that the subsequently described feature or structure may or may not be present, or that the subsequently described event or circumstance may or may not occur, and that the description includes instances where a

particular feature or structure is present and instances where the feature or structure is absent, or instances where the event or circumstance occurs and instances where it does not.

The terms "host microorganism" and "cell" are used interchangeably herein to refer to a living biological cell that can be transformed via insertion of an expression vector. Thus, a host organism or cell as described herein may be a prokaryotic organism (e.g., an organism of the kingdom Eubacteria) or a eukaryotic cell. As will be appreciated by one of ordinary skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus. A preferred prokaryotic cell is *Escherichia coli*. Preferred eukaryotic cells are those derived from fungal, insect, or mammalian cell lines.

The term "heterologous DNA" as used herein refers to a polymer of nucleic acids wherein at least one of the following is true: (a) the sequence of nucleic acids is foreign to (i.e., not naturally found in) a given host microorganism; (b) the sequence may be naturally found in a given host microorganism, but in an unnatural (e.g., greater than expected) amount; or (c) the sequence of nucleic acids comprises two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a heterologous nucleic acid sequence that is recombinantly produced will have two or more sequences from unrelated genes arranged to make a new functional nucleic acid. Specifically, the present invention describes the introduction of an expression vector into a host microorganism, wherein the expression vector contains a nucleic acid sequence coding for an enzyme that is not normally found in a host microorganism. With reference to the host microorganism's genome, then, the nucleic acid sequence that codes for the enzyme is heterologous.

The term "mevalonate pathway" is used herein to refer to the pathway that converts acetyl-CoA to isopentenyl pyrophosphate through a mevalonate intermediate.

The terms "expression vector" or "vector" refer to a compound and/or composition that transduces, transforms, or infects a host microorganism, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. An "expression vector" contains a sequence of nucleic acids (ordinarily RNA or DNA) to be expressed by the host microorganism. Optionally, the expression vector also comprises materials to aid in achieving entry of the nucleic acid into the host microorganism, such as a virus, liposome, protein coating, or the like. The expression vectors contemplated for use in the

present invention include those into which a nucleic acid sequence can be inserted, along with any preferred or required operational elements. Further, the expression vector must be one that can be transferred into a host microorganism and replicated therein. Preferred expression vectors are plasmids, particularly those with restriction sites that have been well documented and that contain the operational elements preferred or required for transcription of the nucleic acid sequence. Such plasmids, as well as other expression vectors, are well known to those of ordinary skill in the art.

The term "transduce" as used herein refers to the transfer of a sequence of nucleic acids into a host microorganism or cell. Only when the sequence of nucleic acids becomes stably replicated by the cell does the host microorganism or cell become "transformed." As will be appreciated by those of ordinary skill in the art, "transformation" may take place either by incorporation of the sequence of nucleic acids into the cellular genome, i.e., chromosomal integration, or by extrachromosomal integration. In contrast, an expression vector, e.g., a virus, is "infective" when it transduces a host microorganism, replicates, and (without the benefit of any complementary virus or vector) spreads progeny expression vectors, e.g., viruses, of the same type as the original transducing expression vector to other microorganisms, wherein the progeny expression vectors possess the same ability to reproduce.

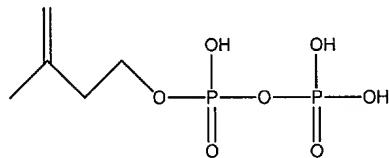
The terms "isolated" or "biologically pure" refer to material that is substantially or essentially free of components that normally accompany it in its native state.

As used herein, the terms "nucleic acid sequence," "sequence of nucleic acids," and variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, and to other polymers containing nonnucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. Thus, these terms include known types of nucleic acid sequence modifications, for example, substitution of one or more of the naturally occurring nucleotides with an analog; internucleotide modifications, such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g.,

aminoalklyphosphoramidates, aminoalkylphosphotriesters); those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.); those with intercalators (e.g., acridine, psoralen, etc.); and those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.). As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB  
5 Commission of Biochemical Nomenclature (*Biochemistry* 9:4022, 1970).

The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the  
10 second sequence.

In a first embodiment, the invention provides a method for synthesizing isopentenyl pyrophosphate, the fundamental building block of isoprenoids, in a host microorganism.



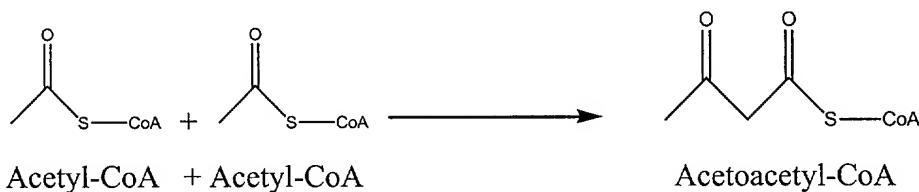
15 Isopentenyl pyrophosphate

Isopentenyl pyrophosphate is also known as "isopentenyl diphosphate" and is commonly abbreviated as "IPP." The method comprises introducing into the host microorganism a plurality of heterologous nucleic acid sequences each coding for a different enzyme in the  
20 mevalonate pathway for producing isopentenyl pyrophosphate. As stated previously, the mevalonate pathway for producing isopentenyl pyrophosphate in living organisms begins with acetyl-CoA and involves a mevalonate intermediate.

In another method for synthesizing isopentenyl pyrophosphate, an intermediate in the mevalonate pathway is introduced into the host microorganism. Although any method for introducing the intermediate may be used, it is preferred to add the intermediate to the culture  
25 medium used to grow the host microorganism. In this way, the intermediate is transported, e.g., via passive diffusion, across the cellular membrane and into the host microorganism.

Either before or after the intermediate is introduced, nucleic acid sequence(s) are introduced that code for those enzymes of the mevalonate pathway necessary to convert the intermediate into isopentenyl pyrophosphate. As will be appreciated by one of ordinary skill in the art, the conversion from the intermediate into isopentenyl pyrophosphate may require one, 5 two, three, or more steps. Although any of the intermediates, i.e., acetyl Co-A, acetoacetyl-CoA, HMG-CoA, mevalonate, mevalonate 5-phosphate, and mevalonate 5-diphosphate, may be used, introduction of DL-mevalonate is a particularly preferred intermediate when using this method in the production of isopentenyl pyrophosphate. Enantiomers of any of the intermediates, such as the bioactive enantiomer D-mevalonate, may 10 be used as well.

As shown in the schematic of FIGS. 1A and 1B, the mevalonate pathway comprises six steps and involves six intermediates. Initially, two molecules of acetyl-coenzyme A (more commonly referred to as "acetyl-CoA") are combined. Acetyl-CoA is produced naturally by the host microorganism when it is in the presence of a suitable carbon source. For example, 15 eukaryotic cells naturally synthesize acetyl-CoA from compounds derived from sugars and fats. An enzyme capable of condensing two molecules of acetyl-CoA to acetoacetyl-CoA is used in this first step of synthesizing isopentenyl pyrophosphate via the mevalonate pathway.



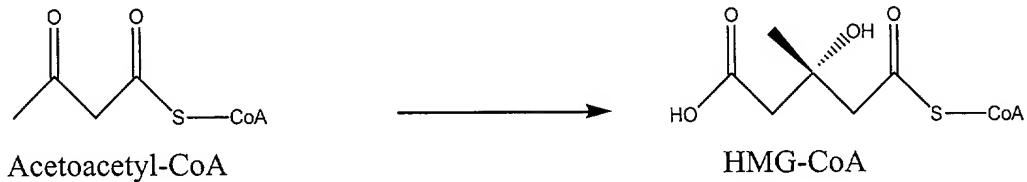
Thus, any DNA fragment coding for an enzyme capable of carrying out this step may be used in the present method. Preferably, however, the DNA fragment codes for an acetoacetyl-CoA 20 thiolase. Genes for such thiolases are known to those of ordinary skill in the art and include, for example, the genes of acetyl-CoA thiolase from *Ralstonia eutrophus* (Peoples et al. (1989), 25 "Poly-β-Hydroxybutyrate Biosynthesis in *Alcaligenes eutrophus* H16" and "Characterization of the Genes Encoding β-Ketothiolase and Acetoacetyl-CoA Reductase," *J. Biol. Chem.* 264 (26): 5293-15297); *Saccharomyces cerevisiae* (*S. cerevisiae*) (Hiser et al. (1994), "ERG10 From

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*Saccharomyces cerevisiae* Encodes Acetoacetyl-CoA Thiolase," *J. Biol. Chem.* 269 (50):

31383-31389); and *Escherichia coli*. It is particularly preferred, however, that the thiolase encoded by the nucleotide sequence of SEQ ID NO 1 be used in the present method.

The next step in the mevalonate pathway requires the condensation of acetoacetyl-CoA, formed from the preceding step, with yet another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This step is catalyzed enzymatically using an enzyme that will condense acetoacetyl-CoA with acetyl-CoA.



Although any DNA fragment that codes for an enzyme capable of carrying out this step may be used, it is preferred that the DNA fragment code for an HMG-CoA synthase. Known genes for HMG-CoA synthases include, without limitation, the synthases from *Blattella germanica* (Martinez-Gonzalez et al. (1993), "3-Hydroxy-3-Methylglutaryl-Coenzyme-A Synthase from *Blattella germanica*. Cloning, Expression, Developmental Pattern and Tissue Expression," *Eur. J. Biochem.* 217(2), 691-699); and *S. cerevisiae*, and thus, are preferred. A particularly preferred synthase is encoded by the nucleotide sequence of SEQ ID NO 2.

The third step converts HMG-CoA to mevalonate. As with the other steps, this conversion is enzymatically controlled.



According to the present method, a DNA fragment coding for an enzyme that is capable of converting HMG-CoA into mevalonate is included in the expression vector. The HMG-CoA reductase genes from *Sulfolobus solfataricus* (Bochar (1997), "3-Hydroxy-3-Methylglutaryl-

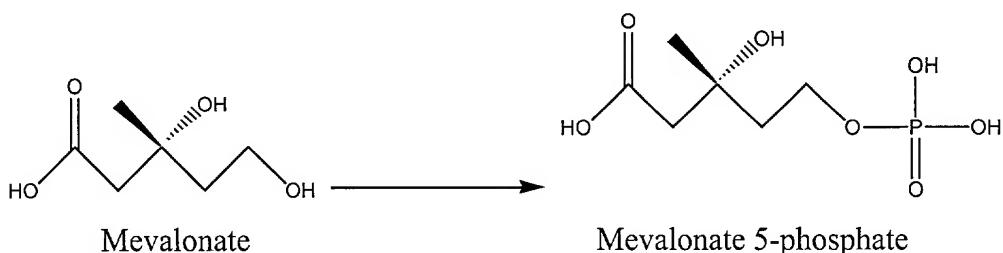
Coenzyme A Reductase of *Sulfolobus solfataricus*: DNA Sequence, Phylogeny, Expression in *Escherichia coli* of the hmgA Gene, and Purification and Kinetic Characterization of the Gene Product," *J. Bacteriol.* 179(11): 3632-3638); *Haloferax volcanii* (Bischoff et al. (1996), "3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase from *Haloferax volcanii*: Purification, Characterization, and Expression in *Escherichia coli*," *J. Bacteriol.* 178(1):19-23); and *S. cerevisiae* (Basson et al. (1988), "Structural and Functional Conservation Between Yeast and Human 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductases, the Rate-Limiting Enzyme of Sterol Biosynthesis," *Mol Cell Biol.* 8(9): 3797-808) are known, and are consequently preferred for the present methods. It is particularly preferred, however, that the nucleotide sequence of SEQ ID NO 3 that encodes an HMG-CoA reductase be used in the present methods.

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The nucleotide sequence defined by SEQ ID NO 3 that encodes an HMG-CoA reductase is a truncated version of the *S. cerevisiae* gene coding for HMG-CoA reductase, *HMG1*. The protein coded for by *HMG1* is an integral membrane protein located in the endoplasmic reticulum of *S. cerevisiae*; it consists of a membrane-spanning, regulatory domain in its N-terminal region (amino acids 1-552) and a catalytically active domain in its C-terminal region. (See Polakowski (1998), "Overexpression of a Cytosolic Hydroxymethylglutaryl-CoA Reductase Leads to Squalene Accumulation in Yeast," *Appl. Microbiol Biotechnol.* 49:66-71.) The nucleotide sequence defined by SEQ ID NO 3 comprises an artificial start codon, followed by nucleotides 1660-3165 of the *HMG1* sequence. Therefore, the nucleotide sequence defined by SEQ ID NO 3 codes for only the catalytically active portion of *S. cerevisiae* HMG-CoA reductase.

The fourth step in the mevalonate pathway involves the enzymatic phosphorylation of mevalonate to form mevalonate 5-phosphate.

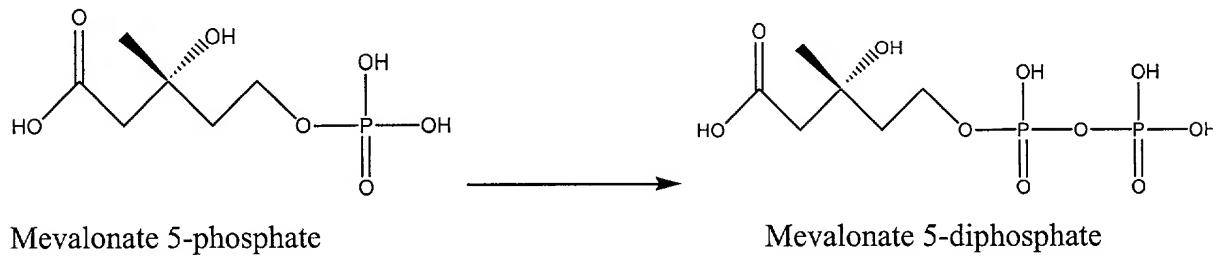
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Although any DNA fragment coding for an enzyme capable of mevalonate phosphorylation may be used, it is preferred that a DNA fragment coding specifically for mevalonate kinase be used. Genes for such kinases are known to those of ordinary skill in the art and include, for example, the mevalonate kinase of *S. cerevisiae* (Oulmouden et al. (1991), "Nucleotide Sequence of the ERG12 Gene of *Saccharomyces cerevisiae* Encoding Mevalonate Kinase," Curr. Genet. 19(1): 9-14). A particularly preferred sequence that codes for this particular kinase is identified in SEQ ID NO 4.

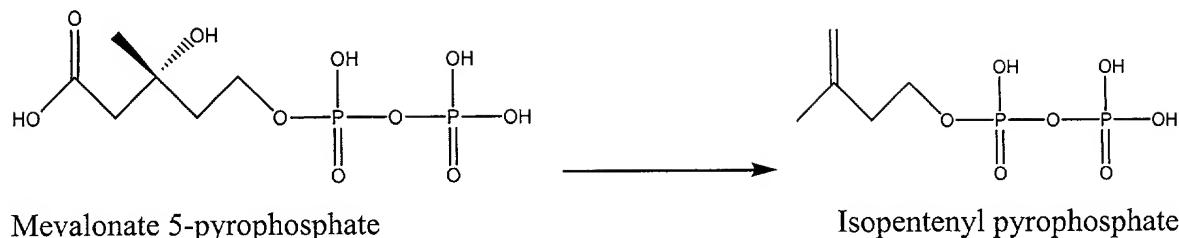
The fifth step in the mevalonate pathway requires the addition of a second phosphate group to mevalonate 5-phosphate. An enzyme catalyzes this step.



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In the present method, a DNA fragment that codes for an enzyme capable of adding a second phosphate group to mevalonate 5-phosphate is used in the expression vector. Preferably, the DNA fragment codes for a phosphomevalonate kinase, such as the gene of the same name obtained from *S. cerevisiae* (Tsay et al. (1991), "Cloning and Characterization of ERG8, an Essential Gene of *Saccharomyces cerevisiae* that Encodes Phosphomevalonate Kinase," *Mol. Cell. Biol.* 11(2):620-31). Such kinases are known to those of ordinary skill in the art and include, for example, the kinase coded by the nucleotide sequence of SEQ ID NO 5.

The sixth and final step of the mevalonate pathway is the enzymatic conversion of mevalonate 5-pyrophosphate into isopentenyl pyrophosphate.



5 Although any DNA fragment coding for a mevalonate pyrophosphate decarboxylase may be used, it is particularly preferred that the gene from *S. cerevisiae* (Toth et al. (1996), "Molecular Cloning and Expression of the cDNAs Encoding Human and Yeast Mevalonate Pyrophosphate Decarboxylase," *J. Biol. Chem.* 271(14):7895-7898) be used. A particularly preferred DNA fragment is the nucleotide sequence of SEQ ID NO 6.

10 When an intermediate is introduced, the method additionally requires introduction of DNA fragments that code for enzymes responsible for catalyzing those steps of the mevalonate pathway located "downstream" from the introduced intermediate. With reference to the mevalonate pathway described above and to the biosynthetic schemes provided in FIGS. 1A and 1B, one of ordinary skill in the art can readily determine which DNA fragments and enzymatic steps are necessary when a given intermediate is introduced into the host microorganism.

15 The mevalonate pathway is contrasted with the mevalonate-independent (or deoxyxylulose-5-phosphate) pathway. In some organisms, isopentenyl pyrophosphate production proceeds by condensation of pyruvate and glyceraldehyde-3-phosphate, via 1-deoxyxylulose-5-phosphate (DXP) as an intermediate. (See Rohmer et al. (1993) *Biochem. J.* 295:517-524.) While some organisms have genes for only one pathway, other organisms have 20 genes for both pathways. For a discussion of both the mevalonate and deoxyxylulose 5-phosphate pathways, reference is made to Lange et al. (2000), "Isoprenoid Biosynthesis: The Evolution of Two Ancient and Distinct Pathways Across Genomes," *Proc. Natl. Acad. Sci. USA* 97(24):13172-13177.

25 Any prokaryotic or eukaryotic host microorganism may be used in the present method so long as it remains viable after being transformed with a sequence of nucleic acids. Generally, although not necessarily, the host microorganism is bacterial. Examples of bacterial host microorganisms include, without limitation, those species assigned to the *Escherichia*,

*Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsielia, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla, and Paracoccus* taxonomical classes. Preferably, the host microorganism is not adversely affected by the transduction of the necessary nucleic acid sequences, the subsequent expression of the proteins (i.e., enzymes), or the resulting 5 intermediates required for carrying out the steps associated with the mevalonate pathway. For example, it is preferred that minimal "cross-talk" (i.e., interference) occur between the host microorganism's own metabolic processes and those processes involved with the mevalonate pathway.

Those of ordinary skill in the art can readily identify suitable host microorganisms. For 10 example, cross-talk is minimized or eliminated entirely when the host microorganism relies exclusively on the "deoxyxylulose 5-phosphate" (or "DXP") pathway for synthesizing isopentenyl pyrophosphate. In such host microorganisms, the mevalonate pathway does not inherently influence (save for the additional synthesis of isopentenyl pyrophosphate) the host microorganism, since it lacks any genes that are equipped to process the proteins (i.e., enzymes) or intermediates associated with the mevalonate pathway. Such organisms relying exclusively 15 or predominately on the deoxyxylulose 5-phosphate pathway include, for example, *Escherichia coli*. Of course, it will be recognized by those of ordinary skill in the art that the host microorganism used in the method may also conduct isopentenyl pyrophosphate synthesis via the mevalonate pathway, either exclusively or in combination with the 20 deoxyxylulose 5-phosphate pathway.

Sequences of nucleic acids coding for the desired enzymes of the mevalonate pathway are prepared by any suitable method known to those of ordinary skill in the art, including, for example, direct chemical synthesis or cloning. For direct chemical synthesis, formation of a polymer of nucleic acids typically involves sequential addition of 3'-blocked and 5'-blocked 25 nucleotide monomers to the terminal 5'-hydroxyl group of a growing nucleotide chain, wherein each addition is effected by nucleophilic attack of the terminal 5'-hydroxyl group of the growing chain on the 3'-position of the added monomer, which is typically a phosphorus derivative, such as a phosphotriester, phosphoramidite, or the like. Such methodology is known to those of ordinary skill in the art and is described in the pertinent texts and literature (e.g., in D.M.

Patent Nos. 5,436,327 and 5,700,637 to Southern et al.). In addition, the desired sequences may be isolated from natural sources by splitting DNA using appropriate restriction enzymes, separating the fragments using gel electrophoresis, and thereafter, recovering the desired nucleic acid sequence from the gel via techniques known to those of ordinary skill in the art, such as utilization of polymerase chain reactions. (See, for example, U.S. Patent No. 4,683,195 to

5 Mullis.)

Once each of the individual nucleic acid sequences necessary for carrying out the desired steps of the mevalonate pathway has been determined, each sequence must be incorporated into an expression vector. Incorporation of the individual nucleic acid sequences may be accomplished through known methods that include, for example, the use of restriction enzymes (such as BamHI, EcoRI, HhaI, XhoI, XmaI, and so forth) to cleave specific sites in the expression vector, e.g., plasmid. The restriction enzyme produces single stranded ends that may be annealed to a nucleic acid sequence having, or synthesized to have, a terminus with a sequence complementary to the ends of the cleaved expression vector. Annealing is performed using an appropriate enzyme, e.g., DNA ligase. As will be appreciated by those of ordinary skill in the art, both the expression vector and the desired nucleic acid sequence are often cleaved with the same restriction enzyme, thereby assuring that the ends of the expression vector and the ends of the nucleic acid sequence are complementary to each other. In addition, DNA linkers may be used to facilitate linking of nucleic acids sequences into an expression vector.

20 A series of individual nucleic acid sequences can also be combined by utilizing methods that are known to those having ordinary skill in the art. (See, for example, U.S. Patent No. 4,683,195 to Minshull et al.)

25 For example, each of the desired nucleic acid sequences can be initially generated in a separate polymerase chain reaction (PCR). Thereafter, specific primers are designed such that the ends of the PCR products contain complementary sequences. When the PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3' ends overlap and can act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are "spliced" together. In this way, a series of individual nucleic acid sequences may be "spliced" together and subsequently transduced into

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a host microorganism simultaneously. Thus, expression of each of the plurality of nucleic acid sequences is effected.

Individual nucleic acid sequences, or "spliced" nucleic acid sequences, are then incorporated into an expression vector. The invention is not limited with respect to the process by which the nucleic acid sequence is incorporated into the expression vector. Those of ordinary skill in the art are familiar with the necessary steps for incorporating a nucleic acid sequence into an expression vector. A typical expression vector contains the desired nucleic acid sequence preceded by one or more regulatory regions, along with a ribosome binding site, e.g., a nucleotide sequence that is 3 - 9 nucleotides in length and located 3 - 11 nucleotides upstream of the initiation codon in *Escherichia coli*. See Shine et al. (1975) *Nature* 254:34 and Steitz, in *Biological Regulation and Development: Gene Expression* (ed. R. F. Goldberger), vol. 1, p. 349, 1979, Plenum Publishing, N.Y., for discussions of ribosome binding sites in *Escherichia coli*.

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Regulatory regions include, for example, those regions that contain a promoter and an operator. A promoter is operably linked to the desired nucleic acid sequence, thereby initiating transcription of the nucleic acid sequence via an RNA polymerase enzyme. An operator is a sequence of nucleic acids adjacent to the promoter, which contains a protein-binding domain where a repressor protein can bind. In the absence of a repressor protein, transcription initiates through the promoter. When present, the repressor protein specific to the protein-binding domain of the operator binds to the operator, thereby inhibiting transcription. In this way, control of transcription is accomplished, based upon the particular regulatory regions used and the presence or absence of the corresponding repressor protein. Examples include lactose promoters (LacI repressor protein changes conformation when contacted with lactose, thereby preventing the LacI repressor protein from binding to the operator) and tryptophan promoters (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor protein has a conformation that does not bind to the operator). Another example includes the *tac* promoter. (See deBoer et al. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80:21-25.) As will be appreciated by those of ordinary skill in the art, these and other expression vectors may be used in the present invention, and the invention is not limited in this respect.

Although any suitable expression vector may be used to incorporate the desired sequences, readily available expression vectors include, without limitation: plasmids, such as pSC101, pBR322, pBBR1MCS-3, pUR, pEX, pMR100, pCR4, pBAD24, pUC19; bacteriophages, such as M13 phage and  $\lambda$  phage; as well as mutant phages, such as  $\lambda$ gt- $\lambda$  $\beta$ . Of course, such expression vectors may only be suitable for a particular host microorganism. One of ordinary skill in the art, however, can readily determine through routine experimentation whether any particular expression vector is suited for any given host microorganism. For example, the expression vector can be introduced into the host organism, which is then monitored for viability and expression of the sequences contained in the vector. In addition, reference may be made to the relevant texts and literature, which describe expression vectors and their suitability to any particular host microorganism.

The expression vectors of the invention must be introduced or transferred into the host microorganism. Such methods for transferring the expression vectors into host microorganisms are well known to those of ordinary skill in the art. For example, one method for transforming *Escherichia coli* with an expression vector involves a calcium chloride treatment wherein the expression vector is introduced via a calcium precipitate. Other salts, e.g., calcium phosphate, may also be used following a similar procedure. In addition, electroporation (i.e., the application of current to increase the permeability of cells to nucleic acid sequences) may be used to transfet the host microorganism. Also, microinjection of the nucleic acid sequence(s) provides the ability to transfet host microorganisms. Other means, such as lipid complexes, liposomes, and dendrimers, may also be employed. Those of ordinary skill in the art can transfet a host microorganism with a desired sequence using these or other methods.

For identifying a transfected host microorganism, a variety of methods are available. For example, a culture of potentially transfected host microorganisms may be separated, using a suitable dilution, into individual cells and thereafter individually grown and tested for expression of the desired nucleic acid sequence. In addition, when plasmids are used, an often-used practice involves the selection of cells based upon antimicrobial resistance that has been conferred by genes intentionally contained within the expression vector, such as the amp, gpt, neo, and hyg genes.

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The host microorganism is transformed with at least one expression vector. When only a single expression vector is used (without the addition of an intermediate), the vector will contain all of the nucleic acid sequences necessary for carrying out isopentenyl pyrophosphate synthesis via the mevalonate pathway. Although such an all-encompassing expression vector may be used when an intermediate is introduced, only those nucleic acid sequence(s) necessary for converting the intermediate to isopentenyl pyrophosphate are required.

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When two versions of an expression vector are used (without the addition of an intermediate), nucleic acid sequences coding for some of the six proteins (i.e., enzymes) necessary for isopentenyl synthesis via the mevalonate pathway may be contained in a first expression vector, while the remainder are contained in a second expression vector. Again, the nucleic acid sequence(s) necessary for converting an introduced intermediate into isopentenyl pyrophosphate will be contained in the expression vector(s). As will be appreciated by those of ordinary skill in the art, a number of different arrangements are possible, and the invention is not limited with respect to the particular arrangement used.

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Once the host microorganism has been transformed with the expression vector, the host microorganism is allowed to grow. For microbial hosts, this process entails culturing the cells in a suitable medium. It is important that the culture medium contain an excess carbon source, such as a sugar (e.g., glucose) when an intermediate is not introduced. In this way, cellular production of acetyl-CoA, the starting material necessary for isopentenyl pyrophosphate production in the mevalonate pathway, is ensured. When added, the intermediate is present in an excess amount in the culture medium.

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As the host microorganism grows and/or multiplies, expression of the proteins (i.e., enzymes) necessary for carrying out the mevalonate pathway, or for carrying out one or more steps within the pathway, is effected. Once expressed, the enzymes catalyze the steps necessary for carrying out the steps of the mevalonate pathway, i.e., converting acetyl-CoA into isopentenyl pyrophosphate. If an intermediate has been introduced, the expressed enzymes catalyze those steps necessary to convert the intermediate into isopentenyl pyrophosphate. Any means for recovering the isopentenyl pyrophosphate from the host microorganism may be used. For example, the host microorganism may be harvested and subjected to hypotonic conditions, thereby lysing the cells. The lysate may then be centrifuged and the supernatant subjected to

high performance liquid chromatography (HPLC). Once the isopentenyl pyrophosphate is recovered, modification may be carried out in the laboratory to synthesize the desired isoprenoid.

If desired, the isopentenyl pyrophosphate may be left in the host microorganism for further processing into the desired isoprenoid *in vivo*. For example, large amounts of the isoprenoid lycopene are produced in *Escherichia coli* specially engineered with the expression vector pAC-LYC, as shown in Examples 3 and 4. Lycopene can be recovered using any art-known means, such as those discussed above with respect to recovering isopentenyl pyrophosphate. Lycopene is an antioxidant abundant in red tomatoes and may protect males from prostate cancer. (See Stahl et al. (1996) *Ach. Biochem. Biophys.* 336(1):1-9.) Of course, many other isoprenoids can be synthesized through other pathways, and the invention is not limited with respect to the particular "downstream" pathway. Thus, the present method not only provides methods for producing isopentenyl pyrophosphate, but offers methods for producing isoprenoids as well.

15      Optionally, when it is desired to retain isopentenyl pyrophosphate in the host microorganism for further biochemical processing, it is preferred that the heterologous nucleic acid sequences introduced into the host microorganism also include a DNA fragment coding for an enzyme capable of converting isopentenyl pyrophosphate to dimethylallyl pyrophosphate. As appreciated by those of ordinary skill in the art, a suitable isomerase will catalyze the conversion of isopentenyl pyrophosphate into dimethylallyl pyrophosphate. Such isomerases are known to those of ordinary skill and include, for example, the isopentenyl pyrophosphate isomerase (*idi*) coded by the nucleotide sequence of SEQ ID NO 10. Isoprenoid biosynthetic pathways require dimethylallyl pyrophosphate, and increased expression of the isomerase ensures that the conversion of isopentenyl diphosphate into dimethylallyl pyrophosphate does not represent a rate-limiting step in the overall pathway.

20      The present methods thus provide for the biosynthetic production of isopentenyl pyrophosphate and isoprenoids derived therefrom. As stated above, isopentenyl pyrophosphate has been available only in relatively small amounts, and the present methods provide a means for producing relatively large amounts of this important compound.

Further, the invention provides the ability to synthesize increased amounts of isoprenoids. As stated above, isoprenoids represent an important class of compounds and include, for example, food and feed supplements, flavor and odor compounds, and anticancer, antimalarial, antifungal, and antibacterial compounds. Preferred isoprenoids are those selected from the group consisting of monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, and steroids. As a class, terpenes are classified based on the number of isoprene units comprised in the compound. Monoterpenes comprise ten carbons or two isoprene units, sesquiterpenes comprise 15 carbons or three isoprene units, diterpenes comprise 20 carbons or four isoprene units, sesterterpenes comprise 25 carbons or five isoprene units, and so forth. Steroids (generally comprising about 27 carbons) are the products of cleaved or rearranged terpenes.

Monoterpenes include, for example, flavors such as limonene, fragrances such as citranellol, and compounds having anticancer activity, such as geraniol. Sesquiterpenes include, without limitation: periplanone B, a cockroach hormone used to lure cockroaches into traps; artemisinin, an antimalarial drug; ginkgolide B, a platelet-activating factor antagonist; forskolin, an inhibitor of adenylyl cyclase; and farnesol, a compound shown to have anticancer activity. Nonlimiting examples of diterpenes include the antibacterial and antifungal compound casbene and the drug paclitaxel. Among triterpenes ( $C_{30}$ ) and tetraterpenes ( $C_{40}$ ) are carotenoids, which are used as antioxidants, coloring agents in food and cosmetics, and nutritional supplements (e.g., as vitamin A precursors). As pathways to these and other isoprenoids are already known, the invention can advantageously be incorporated into an overall scheme for producing relatively large amounts of a desired isoprenoid.

Conveniently, the invention also provides sequences, enzymes, expression vectors, and host cells or microorganisms for carrying out the present methods. For example, the six genes necessary for isopentenyl pyrophosphate synthesis from acetyl-CoA are conveniently provided in SEQ ID NO 7. In addition, the invention also provides sequences for the first three genes and the last three genes in SEQ ID NOs 8 and 9, respectively. These sequences can easily be included in an expression vector using techniques described herein or other techniques well known to those of ordinary skill in the art. In addition, the invention also provides host cells

transformed with one or more of these expression vectors for use in carrying out the present methods.

It is to be understood that, while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

All patents, patent applications, and publications mentioned herein are hereby incorporated by reference in their entireties.

## EXPERIMENTAL

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of the biosynthetic industry and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

In the following examples, efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.), but some experimental error and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees Celsius and pressure is at or near atmospheric pressure at sea level. All reagents, unless otherwise indicated, were obtained commercially.

### Example 1

#### Cloning of the Mevalonate Pathway Operons

##### Assembly of the mevalonate operons

Individual genes for a mevalonate-isoprenoid pathway were assembled to form artificial complete and at least one functional operon. Cloning of the nucleic acid sequences coding for the enzymes of the mevalonate pathway was carried out and the reproduced sequences were divided into two operons. In one of the two operons, the last three genes of the biosynthetic pathway (mevalonate kinase (MK) - SEQ ID NO 4; phosphomevalonate kinase (PMK) - SEQ ID NO 5; and mevalonate pyrophosphate decarboxylase (MPD) - SEQ ID NO 6) were cloned by a polymerase chain reaction (PCR) as one operon by splicing the genes together using overlap extensions (SOEing). This operon is referred to as the mevalonate bottom (MevB) operon (SEQ

5 ID NO 9). In the second of the two operons, the first three genes of the pathway (acetoacetyl-CoA thiolase (*atoB*) - SEQ ID NO 1; HMG-CoA synthase (HMGS) - SEQ ID NO 2; and a truncated version of HMG-CoA reductase (tHMGR) - SEQ ID NO 3) were cloned as a separate artificial operon using the same technique. This operon is referred to as the mevalonate top (MevT) operon (SEQ ID NO 8). The individual genes were isolated by PCR from genomic DNA of *Saccharomyces cerevisiae* and *Escherichia coli* prepared by established microbiologic protocols. (See Sambrook et al., Molecular Cloning: a Laboratory Manual, 3<sup>rd</sup> ed., Cold Harbor Springs Laboratory Press.) The 100  $\mu$ L PCR reactions contained 1X *Pfu* buffer, 1.5 mM MgSO<sub>4</sub> (Stratagene, La Jolla, CA), 200  $\mu$ M of each dNTP (Gibco BRL<sup>TM</sup>, Life Technologies, Inc., Gaithersburg, MD), 500  $\mu$ M of each primer, 100 to 500 ng of template DNA, 5% dimethyl sulfoxide (Sigma, St. Louis, MO), and 2.5 U of *Pfu Turbo* DNA polymerase (Stratagene). The reactions were carried out in a PTC-200 Peltier Thermal Cycler from MJ Research (South San Francisco, CA) with the following temperature cycling program: an initial heating step up to 95°C for four minutes was followed by 30 cycles of 30 seconds of denaturing at 95°C, 30 seconds of annealing at 50°C, and 100 seconds of extension at 72°C, followed by one cycle at 72°C for ten minutes. Once each gene of the operon was amplified from genomic DNA preparations, the operons were assembled by PCR reactions similar to the procedure described above, but using the amplified DNA of all three genes as template DNA and only the forward primer of the outermost 5' gene and the reverse primer of the outermost 3' gene. The assembled operons were isolated on 0.7% agarose gels and purified using a Qiagen gel purification kit (Valencia, CA) according to the manufacturer's instructions.

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## Cloning mevalonate operons into sequencing and expression vectors

As expression of biochemical pathways is often suboptimal from high-copy plasmids containing strong promoters, the artificial mevalonate operon(s) were cloned in a variety of expression vectors to determine the effect of plasmid copy number and promoter strength on expression of the cloned pathway. Prior to testing for pathway expression, the assembled operons were cloned into the pCR4 TOPO vector using the Invitrogen TOPO TA cloning system (Carlsbad, CA) for sequencing purposes. Ligation into pCR4 TOPO vector and transformation of *Escherichia coli* TOP10 cells were carried out according to the manufacturer's instructions. The synthetic operons were excised from the sequenced pCR4 TOPO vectors using restriction

enzymes and ligated into the high-copy vector pBAD24, which contains the arabinose-inducible *araBAD* promoter (Guzman et al. (1995) *J. Bacteriology* 177:4121-4130); pTrc99A, which contains the IPTG-inducible tac promoter (Amann et al. (1988) *Gene* 69:301-315); or into pBBR1MCS-3 (Kovach et al. (1995) *Gene* 166:175-176) or pUC19 (Yanisch-Perron et al. (1985) *Gene* 33:103-119), which contain the unregulated Lac promoters (no plasmid-encoded LacI). The MevB operon was digested with *PstI* and ligated using T4 DNA ligase (New England Biolabs, Inc., Beverly, MA) into the *PstI* site of the low-copy vector, pBBR1MCS-3, containing  $P_{Lac}$  promoter and tetracycline resistance marker. The resulting plasmid, which encodes the enzymes responsible for the conversion of mevalonate to isopentenyl pyrophosphate, was named pBBRMevB. The MevT operon was cloned into the *SalI* site of pBAD24 by digesting with *SalI* restriction enzyme and ligating with T4 DNA ligase. The resulting plasmid, which encodes the enzymes responsible for the conversion of acetyl-CoA to mevalonate, was named pBADMevT.

Addition of isopentenyl pyrophosphate isomerase to MevB operon

The syntheses of geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) require both isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP), to create the backbone structure of all isoprenoids. To ensure sufficient production of DMAPP from IPP, an additional gene, *idi* (encoding isopentenyl pyrophosphate isomerase, SEQ ID NO 10), was amplified by PCR from *Escherichia coli* genomic DNA using primers containing an *XmaI* restriction enzyme site at the 5' ends. Both the amplified product (containing *idi*) and pBBRMevB were digested with *XmaI* and ligated, thereby placing *idi* at the 3' end of the MevB artificial operon. The resulting operon, containing the MevB operon and *idi*, is designated MBI (SEQ ID NO 12). The resulting plasmid (containing the operon of genes that encode for enzymes that convert mevalonate to IPP and DMAPP) was named pBBRMBI-2.

Addition of polyprenyl pyrophosphate synthase(s) to MBI operon

In order to direct products of the mevalonate pathway operons to the different classes of isoprenoids (monoterpenes, sesquiterpenes, diterpenes, etc.), various polyprenyl pyrophosphate synthases were cloned into the MBI operon, such as geranyl diphosphosphate (GPP) synthase, farnesyl pyrophosphate (FPP) synthase, and geranylgeranyl pyrophosphate (GGPP) synthase. Polyprenyl pyrophosphate synthases were cloned by PCR using forward primers with a *SacII*

restriction site and reverse primers with a *SacI* restriction site. Using restriction enzymes and T4 DNA ligase, the polyprenyl pyrophosphate synthases were cloned between the *SacII* and *SacI* sites of pBBRMBI-2. For example, farnesyl pyrophosphate synthase gene *ispA* (SEQ ID NO 11) was isolated by PCR from *Escherichia coli* genomic DNA and cloned between the *SacII* and *SacI* sites of pBBRMBI-2, 3' of *idi* and the MevB operon. The resulting operon, containing the MevB operon, *idi*, and *ispA* (SEQ ID NO 11) has been designated MBIS (SEQ ID NO 13). The plasmid, which encodes the enzymes responsible for the synthesis of farnesyl pyrophosphate (FPP) from mevalonate, was named pBBRMBIS-2.

### Example 2

#### Functionality of the Engineered Mevalonate Operon(s) by Growth/No-Growth Phenotype

Functionality of the various genetic constructs was shown by expression of the artificial mevalonate-isoprenoid pathway. The plasmids were introduced into an *Escherichia coli* host in which the mevalonate-independent (DXP) isoprenoid pathway was inactivated. *Escherichia coli* strain DMY1 (Kuzuyama et al. (1999) *Biosci. Biotechnol. Biochem.* 63:776-778) contains a mutation (insertion/deletion) in the gene encoding for 1-deoxyxylulose 5-phosphate reductoisomerase (or DXR, the second step of the DXP pathway) that causes inactivation of the mevalonate-independent pathway. Since this mutation is lethal to *Escherichia coli*, the strain must be propagated in Luria-Bertoni (LB) medium (available from, for example, Sigma, St. Louis, MO) containing 0.5 mM of methylerithrytol (ME), the product of DXR; or it must have an alternate pathway for the production of isopentenyl pyrophosphate.

Cultures of *Escherichia coli* strain DMY1 were made electrocompetent according to the method of Sambrook et al. (above) and transformed with pBBRMBI-2, or both pBBRMBI-2 and pBADMevT. Newly transformed DMY1 cells were first allowed to recover on LB agar plates overnight, and were supplemented with 0.5 mM ME and appropriate antibiotics at 37°C prior to testing growth on media devoid of ME. DMY1 cells transformed with only pBBRMBI-2 were plated on LB agar devoid of ME, but supplemented with 1 mM DL-mevalonate prepared by incubating 1 volume of 2 M DL-mevalonic acid lactone (Sigma, St. Louis, MO) with 1.02 volumes of 2 M KOH at 37°C for 30 minutes. DMY1 cells transformed with both pBBRMBI-2

and pBADMevT plasmids were plated on LB agar with antibiotics only (no ME or DL-mevalonate). All test plates were incubated for 48 hours at 37°C.

5 *Escherichia coli* strain DMY1 cells containing pBBRMBI-2 were able to grow on LB agar plates with 1 mM DL-mevalonate, whereas *Escherichia coli* DMY1 cells without the plasmid or with pBBR1MCS-3 (empty vector control) did not grow. The MBI operon successfully converted the supplemented mevalonate to isopentenyl pyrophosphate and dimethylallyl pyrophosphate, thereby complementing the *dxr* deletion.

10 *Escherichia coli* strain DMY1 cells containing pBADMevT and pBBRMBI-2 were able to grow on LB agar plates not supplemented with DL-mevalonate, whereas *Escherichia coli* DMY1 cells without either of the plasmids could not grow on LB agar alone. The expression of the MevT and MBI operons successfully converted acetyl-CoA to isopentenyl pyrophosphate and dimethylallyl pyrophosphate *in vivo*, thereby restoring growth to *Escherichia coli* strain DMY1, in which the native DXP isoprenoid pathway is inactive.

15 **Example 3**

**Production of Carotenoids from Mevalonate Using the MBI Artificial Operon**

20 The production of a carotenoid was used to demonstrate the benefits of expressing the artificial mevalonate-dependent IPP biosynthetic pathway over the native *Escherichia coli* DXP-isoprenoid pathway. The increased productivity of the mevalonate-dependent isopentenyl pyrophosphate biosynthetic pathway encoded by the synthetic operons was assayed by coupling isopentenyl pyrophosphate production to the production of lycopene. This was accomplished by co-transforming *Escherichia coli* with pAC-LYC, a low-copy broad-host plasmid that expresses the genes encoding the pathway for lycopene production from farnesyl pyrophosphate. The genes expressed from pAC-LYC are *crtE* (geranylgeranyl pyrophosphate synthase), *crtB* (phytoene synthase), and *crtI* (phytoene desaturase) from *Erwinia herbicola*, which were cloned into pACYC184 using methods similar to those described in Examples 1 and 2. *Escherichia coli* naturally produces farnesyl pyrophosphate from two molecules of isopentenyl pyrophosphate and one molecule of dimethylallyl pyrophosphate through the enzyme farnesyl pyrophosphate synthase, *ispA* (SEQ ID NO 11). Alternatively, more flux can be directed from

the mevalonate pathway to the lycopene pathway by including the *Escherichia coli* gene encoding farnesyl pyrophosphate synthase into the artificial operon(s).

From previous experiments (not described herein), it was found that the production of isopentenyl pyrophosphate from the mevalonate pathway operons was greater in the *Escherichia coli* strain DH10B than in the *Escherichia coli* strain DMY1. In order to demonstrate isopentenyl pyrophosphate production from the mevalonate pathway only, the gene encoding 1-deoxyxylulose 5-phosphate reductoisomerase, *dxr*, was inactivated in *Escherichia coli* strain DH10B by the method detailed by Datsenko et al. (2000), "One-step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products," *PNAS* 97:6640-6645. In the resulting *Escherichia coli* strain, named DPDXR1, the mevalonate independent pathway (or DXP pathway) is inactive, and in order to survive, the strain must either be propagated in LB medium containing 0.5 mM of methylerithrytol (ME) or have an alternate pathway for the production of isopentenyl pyrophosphate.

*Escherichia coli* strain DPDXR1 was transformed with pAC-LYC and pBBRMBI-2, while *Escherichia coli* strain DH10B was transformed with pAC-LYC and pBBR1MCS-3 (control) by electroporation. Transformants were selected on LB agar plates supplemented with 50  $\mu$ g/ml chloramphenicol, 10  $\mu$ g/ml tetracycline, and 1 mM DL-mevalonate by incubating overnight at 37°C. One colony of each strain (*Escherichia coli* DPDXR1 harboring pAC-LYC and pBBRMBI-2 or *Escherichia coli* DH10B harboring pAC-LYC and pBBR1MCS-3) was transferred from the LB agar selection plate to 5 ml of LB liquid medium also supplemented with 50  $\mu$ g/ml chloramphenicol, 10  $\mu$ g/ml tetracycline, and 1 mM DL-mevalonate. These seed cultures were incubated at 37°C until they reached a stationary growth phase. The cell density of each seed culture was determined by measuring the optical density of the culture at a wavelength of 600 nm (OD<sub>600</sub>). These seed cultures were then used to inoculate 5 ml test cultures of LB medium with appropriate antibiotics and increasing concentrations of DL-mevalonate. The volume of seed culture used to inoculate each fresh 5 ml culture was calculated to give an initial OD<sub>600</sub> value of 0.03. Test cultures were incubated for 48 hours at 30°C, after which growth was arrested by chilling the cultures on ice. The optical density of each culture was measured. One ml of each culture was harvested by centrifugation (25000 x g, 30 seconds), the supernatant was removed, and cell pellets were suspended in 500  $\mu$ L of acetone

by rapid mixing with a Fisher Vortex Genie 2™ mixer (Scientific Industries, Inc., Bohemia, NY). The acetone/cell mixtures were incubated at 55°C for 10 minutes to aid in the extraction of lycopene from the cells. Extracted samples were centrifuged (25000 x g, 7 minutes) to remove cell debris, and the cleared acetone supernatants were transferred to fresh tubes. The 5 lycopene concentration of each acetone extraction was assayed by absorbance at 470 nm using a Beckman™ DU640 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) and a 400 µL quartz cuvette. Absorbance values at 470 nm were converted to lycopene concentrations using linear regressions from a standard curve produced using pure lycopene (Sigma, St. Louis, MO). Final lycopene concentrations of each strain at increasing concentration of substrate is reported 10 in FIG. 2. As shown in FIG. 2, lycopene production as a function of substrate concentration following shaking for 48 hours at 30°C demonstrated that lycopene produced from natural levels of isopentenyl pyrophosphate in non-engineered *Escherichia coli* strain DH10B (vertical stripes) remains relatively constant, while lycopene produced from isopentenyl pyrophosphate generated by engineered *Escherichia coli* strain DPDXR1 harboring the plasmid, pBBRMBI-2 (horizontal stripes), significantly increases at mevalonate substrate concentrations of 10 mM and higher. 15

#### Example 4

##### Production of Carotenoids from Luria-Bertoni Broth

##### Using the Complete Mevalonate Pathway

20 It was demonstrated that significantly higher levels of isopentenyl pyrophosphate and isoprenoids derived therefrom were produced using the complete mevalonate-isoprenoid operon when compared to the native DXP pathway. The complete mevalonate-isoprenoid pathway was expressed using the two operons, MevT and MBI, which were expressed from pBADMevT and pBBRMBI-2, respectively, and coupled to pAC-LYC to demonstrate the *in vivo* production of 25 the carotenoid lycopene, using precursors produced by primary cellular metabolism.

30 *Escherichia coli* strain DH10B was transformed with pBADMevT, pBBRMBI-2, and pAC-LYC by electroporation. Transformants were selected on LB agar plates containing 50 µg/ml carbenicillin, 10 µg/ml tetracycline, and 50 µg/ml chloramphenicol. A single colony of the strain was transferred from the LB agar plate to 5 ml of LB liquid medium containing the same antibiotics. This seed culture was incubated by shaking at 37°C until growth reached a

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stationary phase. The cell density of each seed culture was measured at OD<sub>600</sub>, and the cells were used to inoculate 5 ml test cultures of fresh LB medium plus the same antibiotics to give an OD<sub>600</sub> of 0.03. The test cultures were incubated for 48 hours at 30°C, after which growth was arrested by chilling the cultures on ice. The remainder of the experimental procedure was followed as described in Example 3. Final lycopene production (μg/ml lycopene per OD<sub>600</sub>) of the pBADMevT, pBBRMBI-2, pAC-LYC plasmid system was compared to the lycopene production from pAC-LYC plasmid only (control) in the *Escherichia coli* DH10B strain, as shown in FIG. 3. This figure illustrates, in graph form, the amount of lycopene produced for each strain, normalized for cell density, after shaking for 48 hours at 30°C. The column on the left represents the amount of lycopene produced naturally in a non-engineered *Escherichia coli* strain (containing only pAC-LYC as a control). The column on the right represents the amount of lycopene produced from an *Escherichia coli* strain engineered to overproduce isopentenyl pyrophosphate from the mevalonate-isoprenoid pathway.

### Example 5

#### Production of Terpenes by Coupling of Artificial Mevalonate Operon(s) to Terpene Cyclases

Many valuable natural products were produced from the isoprenoid biosynthetic pathways described herein. Depending on the desired isoprenoid, the described operon(s) were modified, and/or additional operons or other means for chemical synthesis were provided to produce the precursors for the various classes. The following experiments demonstrated the synthesis of sesquiterpenes using the farnesyl pyrophosphate synthase, *ispA* (SEQ ID NO 11), as well as the means by which other classes of isoprenoids, such as diterpenes, were synthesized by varying the synthase cloned into the operon(s) to create the desired precursor.

#### In vivo Production of Sesquiterpenes

Amorphadiene, a precursor to the antimalarial drug artemisinin, was produced from the co-expression of the mevalonate-isoprenoid pathway, along with a sesquiterpene cyclase-encoding amorphadiene synthase. The MBIS operon expressed from pBBRMBIS-2 was coupled with amorpha-4,11-diene synthase (ADS) for the *in vivo* production of the sesquiterpene amorphadiene in *Escherichia coli*.

A gene coding for amorpha-4,11-diene synthase (ADS) was constructed so that, upon translation, the amino acid sequence would be identical to that described by Merke et al. (2000) *Ach. Biochem. Biophys.* 381(2): 173-180. The ADS gene contains recognition sequences 5' and 3' of the coding DNA corresponding to the restriction endonucleases *Nco*I and *Xma*I, 5 respectively. The ADS gene was digested to completion with the restriction endonucleases, along with DNA for the plasmid pTrc99A. The 1644-bp gene fragment and the 4155-bp plasmid fragment were purified using 0.7% agarose gels and a Qiagen gel purification kit (Valencia, CA) according to the manufacturer's instructions. The two fragments were then ligated using T4 DNA ligase from New England Biolabs (Beverly, MA), resulting in plasmid 10 pTRCADS. The insert was verified by sequencing to be the amorpha-4,11-diene synthase gene.

*Escherichia coli* strain DH10B was transformed with both the pBBRMBIS-2 and pTRCADS plasmids by electroporation. Bacterial colonies were then grown on Luria-Bertoni (LB) agar containing 50  $\mu$ g/ml carbenicillin and 10  $\mu$ g/ml tetracycline. A single bacterial colony was transferred from the agar plates to 5 ml LB liquid medium containing the same antibiotics and cultured by shaking at 37°C for 16-18 hours. Five hundred  $\mu$ L of this culture 15 was transferred into 5 ml fresh LB liquid medium with the same antibiotics, then cultured by shaking at 37°C to an optical density of 0.816 at 600 nm (OD<sub>600</sub>). A 1.6 ml portion of this culture was used to inoculate a flask containing 100 ml of LB liquid medium with 50  $\mu$ g/ml carbenicillin and 10  $\mu$ g/ml tetracycline, which was cultured by shaking at 37°C. After 1.5 20 hours, 1 ml of 1 M mevalonate and 100  $\mu$ L of 500 mM isopropylthio- $\beta$ -D-galactoside (IPTG) were added to the culture, and it continued to be shaken at 37°C. Amorpha-4,11-diene concentration was determined by extracting 700  $\mu$ l samples (taken hourly) with 700  $\mu$ l of ethyl 25 acetate in glass vials. The samples were then shaken at maximum speed on a Fisher Vortex Genie 2™ mixer (Scientific Industries, Inc., Bohemia, NY) for three minutes. The samples were allowed to settle in order to separate the ethyl acetate-water emulsions. Prior to gas chromatography-mass spectrometry analysis, the ethyl acetate layer was transferred with a glass Pasteur pipette to a clean glass vial.

Ethyl acetate culture extracts were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS). A 1  $\mu$ l sample was separated on the GC using a

DB-5 column (available from, for example, Agilent Technologies, Inc., Palo Alto, CA) and helium carrier gas. The oven cycle for each sample was 80°C for two minutes, increasing temperature at 30°C/minute to a temperature of 160°C, increasing temperature at 3°C/min to 170°C, increasing temperature at 50°C/minute to 300°C, and a hold at 300°C for two minutes. The resolved samples were analyzed by a Hewlett-Packard model 5973 mass selective detector that monitored ions 189 and 204 m/z. Previous mass spectra demonstrated that the amorpha-4,11-diene synthase product was amorphadiene and that amorphadiene had a retention time of 7.9 minutes using this GC protocol. Since pure standards of amorpha-4,11-diene are not available, the concentrations must be quantified in terms of caryophyllene equivalence. A standard curve for caryophyllene has been determined previously, based on a pure standard from Sigma (St. Louis, MO). The amorpha-4,11-diene concentration is based on the relative abundance of 189 and 204 m/z ions to the abundance of the total ions in the mass spectra of the two compounds.

The amorphadiene concentration of the cultures seven hours after the addition of IPTG and mevalonate is shown in FIG. 4. The figure shows the concentration of amorphadiene produced seven hours after the addition of mevalonate and isopropylthio- $\beta$ -D-galactoside (IPTG). The column on the left shows the concentration of amorphadiene produced from non-engineered *Escherichia coli* harboring the pTRCADS plasmid alone. The column on the right shows the concentration of amorphadiene produced from engineered *Escherichia coli* harboring the pBBRMBIS-2 and pTRCADS plasmids. The *Escherichia coli* strain engineered to make farnesyl pyrophosphate from the mevalonate isoprenoid pathway produced 2.2  $\mu$ g/ml amorphadiene, whereas the non-engineered strain (without the mevalonate isoprenoid pathway) produced only 0.13  $\mu$ g/ml.

## *In vivo* Production of Diterpenes

The plasmid pBBRMBIS-2 was modified to include a gene encoding geranylgeranyl pyrophosphate synthase (instead of farnesyl pyrophosphate synthase). To demonstrate the utility of the artificial mevalonate-isoprenoid for *in vivo* diterpene production, this modified expression system was coupled with a plasmid expressing casbene synthase. Casbene synthase cDNA cloned into expression vector pET21-d (Hill et al. (1996), *Arch Biochem. Biophys.* 336:283-289) was cut out with *Sall* (New England Biolabs, Beverly, MA) and *Nco*I (New

England Biolabs, Beverly, MA) and re-cloned into high-copy-number expression vector pTrc99A. The gene fragment and the plasmid fragment were purified with 0.7% agarose gels using a Qiagen gel purification kit (Valencia, CA) according to the manufacturer's instructions. The two fragments were then ligated using T4 DNA ligase from New England Biolabs (Beverly, MA), resulting in plasmid pTrcCAS.

*Escherichia coli* strain DH10B was transformed with both the modified pBBRMBIS-2 and pTrcCAS plasmids by electroporation. Bacterial colonies were then grown on Luria-Bertoni (LB) agar containing 50 µg/ml carbenicillin and 10 µg/ml tetracycline. A single bacterial colony was transferred from the agar plates to 5 ml LB liquid medium containing the same antibiotics and cultured by shaking at 37°C for 16-18 hours. Five hundred microliters of this culture was transferred into 5 ml fresh LB liquid medium with 50 µg/ml carbenicillin and 10 µg/ml tetracycline, and cultured by shaking at 37°C to an optical density of 0.816 at 600 nm (OD<sub>600</sub>). A 150 µL portion of this culture was used to inoculate a flask containing 25 ml of LB liquid medium with 50 µg/ml carbenicillin, 10 µg/ml tetracycline, and 20 mM mevalonate. This mixture was cultured by shaking at 37°C. After 1.5 hours, 250 µL of 100 mM IPTG were added to the culture, and it continued to be shaken at 37°C. Casbene concentration of the culture was determined hourly by extracting 450 µL samples. To these samples was added 450 µL of ethyl acetate in a glass vial. The samples were then shaken on a Fisher Vortex Genie 2™ mixer (Scientific Industries, Inc., Bohemia, NY) for three minutes. The samples were allowed to settle in order to separate the ethyl acetate-water emulsion. The ethyl acetate layer was transferred with a glass Pasteur pipette to a clean vial.

Ethyl acetate culture extracts were analyzed on a Hewlett-Packard 6890 gas chromatograph/mass spectrometer (GC/MS). A 1  $\mu$ l sample was separated on the GC using a DB-5 column (available from, for example, Agilent Technologies, Inc., Palo Alto) and helium carrier gas. The oven cycle for each sample was 80°C for two minutes, increasing temperature at 10°C/minute to a temperature of 300°C, and a hold at 300°C for two minutes. The resolved samples were analyzed by a Hewlett-Packard model 5973 mass selective detector that monitored ions 229, 257, and 272 m/z. Previous mass spectra had demonstrated that the casbene synthase product was casbene and that casbene had a retention time of 16.6 minutes using this GC

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protocol. FIG. 5 shows the gas chromatographic analysis and resulting GC/MS chromatogram for the ethyl acetate extracts taken seven hours after addition of IPTG from *Escherichia coli* engineered to produce isoprenoids from the artificial modified MBIS operon, thereby expressing the casbene cyclase from the pTrcCAS plasmid. As a reference, FIG. 6 shows the spectrogram for casbene.